

Laboratory Procedure Manual

Analyte: **Syphilis**

Matrix: **Serum**

Method: **Serodia *Treponema pallidum*
Particle Agglutination (TP-PA) Test**

Method No.:

Revised:

as performed by: *Division of HIV, STD and TB Laboratory Research
National Center for Infectious Diseases*

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Important Information for Users

CDC periodically refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for NHANES 2003–2004 data.

A tabular list of the released analytes follows:

Lab Number	Analyte	SAS Label
I36_c	LBDSY4	<i>Treponema pallidum</i> Particle Agglutination (TP-PA)

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The Serodia *Treponema pallidum* particle agglutination (TP-PA) test is a treponemal test for the serologic detection of antibodies to the various species and subspecies of pathogenic *Treponema*, the causative agents of syphilis, yaws, pinta, bejel, and endemic syphilis. The test is a passive agglutination procedure based on the agglutination of gel particles sensitized with *T. pallidum* antigens by antibodies found in the patient's serum (1–3). The test is intended as a confirmatory test to replace the microhemagglutination assay for antibodies to *T. pallidum* (MHA-TP).

Serum containing antibodies to pathogenic treponemes react with gel particles sensitized with sonicated *T. pallidum*, Nichols strain (the antigen), to form a smooth mat of agglutinated gel particles in the microtiter tray well. If antibodies are not present, the particles settle to the bottom of the tray well, forming a characteristic compact button of unagglutinated particles. The unsensitized gel particle control well for each serum should also show this compact button, or the absence of agglutination.

The TP-PA test is used to confirm the reactive results (2, 3) of a nontreponemal screening test for syphilis, such as the Venereal Disease Research Laboratory (VDRL) slide test, or as a diagnostic test in patients with a nonreactive nontreponemal test but with signs or symptoms suggestive of late syphilis.

2. SAFETY PRECAUTIONS

The risk of infection due to an occupational exposure to blood depends upon the prevalence of blood-borne pathogens in the population supplying the blood specimens, the probability of infection given a particular type of exposure to a blood-borne pathogen, and the frequency of exposures (4, 5).

T. pallidum is present in circulating blood during primary and secondary syphilis. The minimum number (LD50) of *T. pallidum* organisms needed to infect by subcutaneous injection is 23 (6). The concentration of *T. pallidum* in patients' blood during early syphilis, however, has not been determined. The ability of blood inoculated with *T. pallidum* to infect animals is reduced by refrigerated storage (7, 8). Although multiple instances of transmission of *T. pallidum* due to transfusion of an infected donor's blood were reported prior to the introduction of penicillin for treatment of syphilis and of refrigeration for blood storage (7). Subsequent reports have been rare (7, 8). Infection of a health care or laboratory worker following exposure to *T. pallidum* infected blood has, apparently, not been reported.

Authoritative sources focus attention on infection with hepatitis B virus (HBV), hepatitis C virus (HCV), and human immunodeficiency virus (HIV) as the principal concerns associated with exposure to blood (5, 10–13). The prevalence of these infections varies greatly among patient populations tested for *T. pallidum* infection. HBV infection is most common. HBV viremia is indicated by tests for HBV surface antigen (HBsAg) in serum. Prevalence of anti-HBsAg, from published studies of patients in hospitals and emergency rooms cited in a recent review, ranged from 0.9% to 6% (4, 14–17). Unlike initial HBV infection, in which only a minority of individuals continues to be viremic, initial HCV and HIV infections lead to persistent viremia in most individuals. Consequently, serum antibody to HCV and HIV are indicators of potential infectiousness. Seroprevalences of antibody to HCV in studies of patients in hospitals and emergency rooms cited in a recent review ranged from 2 to 18% (13, 16–19). HIV prevalence ranged from 0.1% to 5.6% in patients enrolled in a national hospital surveillance system (4, 20). All three infections are more common among patients at increased risk for syphilis, especially patients with a history of illegal drug use. For example, seroprevalences of antibody to HCV were 10% among non-drug-using attendees at sexually transmitted diseases clinics and 60% among injection-drug users (21–23).

While infections with HBV (22, 24) and HIV (12, 25–27) can occur with skin and mucous membrane exposures to blood, needle stick and percutaneous injury with blood-coated sharp objects are the principal sources of laboratory associated acquisition of these agents. The risk of infection following exposure to blood from an infected patient is greatest for HBV, except for exposed individuals who are immune due to prior HBV infection or vaccination. The risk is highest if the source individual is HBsAg-positive (22, 28–30) and is positive for envelope (E) antigen. A vaccine to prevent HBV infection has been available since 1982 and is strongly recommended for health care workers with potential exposures to blood or other body fluids (28, 31, 32). Individuals with anti-HBV antibody from vaccination or prior infection are considered to be immune to HBV infection.

The risk of HCV infection due to needle-stick exposure to blood from an individual with antibody to HCV was 10% in one study (22, 33, 34), but HCV does not appear to survive long in serum held at room temperature (22, 35). A vaccine is not yet available to immunize against HCV infection. Repeated infection with HCV appears to be possible in spite of detectable serum anti-HCV antibody, although the significance of reinfection is unknown (21, 36, 37).

The risk of infection with HIV following a single needle-stick exposure to blood from a patient known to be infected with HIV is approximately 0.3% (4). The risks following mucous membrane or skin exposures to HIV-infected blood average approximately 0.1 and <0.1%, respectively (12, 25, 27, 38). The lower rate of transmission for HIV than for HBV or HCV probably reflects a lower concentration of HIV in the blood of infected persons. A vaccine is not available to immunize against HIV infection. The frequency and significance of repeated exposures of individuals with prior anti-HIV antibody is unknown.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

- A. Each shipment of specimens received from the NHANES III mobile unit contains a corresponding transmittal sheet and an ASCII data file (KOUTPUT.TXT) on a 3 ½ " high density floppy diskette. The data file, containing the specimen ID, collection date, and type of sample (i.e., whole blood, serum, plasma) is checked against the information on the transmittal sheet and specimen label prior to the assay.
- B. After the data is calculated and the final values are approved by the reviewing supervisor for release, all results are entered onto the NHANES diskettes by using the program provided by National Center for Health Statistics (NCHS).
- C. After the results are entered on diskettes, back up copies are made and stored in locked areas.
- D. The original diskette containing analytical results is mailed to NCHS.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

- A. No special instruction such as special diet or fasting is necessary.
- B. Fresh serum samples are the specimens of choice for the Serodia TP-PA test. Serum specimens may be collected using regular red-top or serum separator Vacutainers. Specimens are allowed to clot at room temp and centrifuged.
- C. Transfer serum to 2-mL polypropylene screw-capped vials. Freeze at $\leq -20^{\circ}\text{C}$.
- D. Each week, batches of frozen serum samples are placed in a Styrofoam-insulated shipping container with dry ice and sent to the laboratory by an overnight courier.
- E. Serum specimens are stable up to 72 hours at $4-8^{\circ}\text{C}$. For longer periods, store the serum at $\leq -20^{\circ}\text{C}$ in glass or plastic vials, as long as the vials are tightly sealed to prevent desiccation of the sample.
- F. Excessively hemolyzed, contaminated, or lipemic sera may give aberrant results and should not be used. A specimen is too hemolyzed for testing when printed material cannot be read through it. Heat-inactivated sera may be used (56°C for 30 minutes). Excessive inactivation time or temperature may increase nonspecific background activity which could result in equivocal results.
- G. The optimal amount of serum is 0.5 to 1.0 mL. Specimen volumes of less than 0.4 mL are not acceptable.
- H. Avoid repeated freeze-thawing cycles, which may compromise specimen integrity.
- I. Specimens should generally arrive frozen.
- J. Residual samples are frozen at $\leq -20^{\circ}\text{C}$.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

A. Instrumentation

- (1) Micropipettes to deliver 5 to 200 μL .
- (2) Automatic vibratory shaker

B. Other Materials

- (1) Droppers to deliver 25 μL for delivery of sensitized and nonsensitized particles.
- (2) Disposable, clear plastic trays with 8 rows of 12 U-shaped wells each.
- (3) 1.0- or 2.0-mL serologic pipettes graduated in 1/100 mL.
- (4) Safety pipetting devices for serologic pipettes.
- (5) Tray viewer.
- (6) 2-mL and 10-mL serologic pipettes
- (7) Latex gloves, safety glasses, and protective clothing.
- (8) Discard container and disinfectant.

C. Reagent Preparation

Each Serodia TP-PA kit contains enough reagents to test 92 samples and the controls. Reagents should be mixed gently to avoid possible deterioration of the antigen-carrier complex. Reagents are stable until the expiration date printed on the label. All reagents should be stored at 4–8°C.

(1) Sensitized particles

Lyophilized preparation of colored gelatin particles coated with *Treponema pallidum*. Rehydrate each 20 X 5 test kit vial with 0.6 mL of reconstituting solution. Reconstituted suspension contains a 1.0% suspension of sensitized gelatin particles and 0.08% (w/v) sodium azide.

(2) Unsensitized particles

Lyophilized preparation of colored gelatin particles that are not sensitized with *T. pallidum*. Rehydrate each 5 × 20 test kit vial with 0.6 mL of reconstituting solution. Lyophilized reagent when rehydrated contains 1.0% suspension of unsensitized gelatin particles and 0.08% (w/v) sodium azide.

(3) Reconstituting solution (liquid).

Aqueous solution of 0.05M phosphate buffered saline (PBS) containing 0.2M sodium chloride (NaCl), 0.6% normal rabbit serum, and 0.06% sodium azide. The solution is used to reconstitute the Sensitized and Unsensitized Particles.

(4) Sample diluent (liquid).

Aqueous solution of 0.05M PBS containing 0.9M NaCl, 2% normal rabbit serum, 0.1% rabbit testicular extract, and 0.1% sodium azide at pH 6.7–7.3 used to dilute serum specimens.

(5) Reactive control (liquid).

Serum containing rabbit antibodies to *T. pallidum* and should demonstrate a titer of 1:320 final dilution, ± 1 doubling dilution when tested according to the procedure prescribed by the manufacturer. Contains 0.099% sodium azide as preservative.

(6) Nonreactive control (liquid).

The Nonreactive control is made from normal donor serum that is nonreactive in the tests for syphilis. The nonreactive control is treated as a patient sample and should be included in each batch of patient samples to ensure proper and consistent performance of the assay. Contains 0.1% sodium azide as preservative.

D. Preparation of Control Serum Samples

(1) Positive Control Serum

Prepared from rabbit serum samples containing antibodies to *T. pallidum*. Serum contains 0.099% sodium azide and is ready to use. Bring to room temp before use.

(2) Nonreactive Control Serum

Prepared from human serum samples free of *T. pallidum* antibodies. Serum contains 0.1% sodium azide and is ready to use. Bring to room temp before use.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Working Standards

The reactive control is used to determine level of reactivity of the test for lot to lot comparison and as an indication of whether reagents are deteriorating. Reactive control should have a titer of 1:320 ± 1 doubling dilution.

B. Pipettors and Tips

With the pipettors currently available, the measurement of small serum volumes is routine. Most manufacturers include in the specifications of the pipettors the accuracy for frequently used microliter volumes. Daily use may affect pipettors, making them lose their initial accuracy. The difference in disposable tips from sources other than the pipette manufacturer is probably the most common error. For budgetary reasons, a less expensive brand of pipette tips may be substituted for those of the manufacturer. Although the less expensive brand may be satisfactory, the laboratory should verify the accuracy of the substitute pipette tips in their system. Commercial kits to check the accuracy are available. Also, manufacturers provide procedures for checking the accuracy of their equipment. Historically, the gravimetric or spectrophotometric procedures, which use the weight of water or absorbance of a substance at a given wavelength, have been the most accepted methods used to calibrate pipettors. These procedures should not be used instead of those specified by the manufacturer nor do they substitute for an annual verification and repair by a company qualified to do this.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

A. Preliminaries

- (1) Bring all reagents and serum samples to room temp before beginning test.
- (2) The reactive control should be titered every day that samples are tested. The positive and negative controls should be included in every run.

B. Sample Preparation

Samples are ready to be diluted the same as all patient samples.

C. Test Procedure

- (1) Allow all reagents to reach room temperature.

- (2) The kit control sera should be included in each run. The reactive control should be titrated each day that samples are tested.
In the first row of the microplate, place 100 µL of diluent buffer in well #1, and 25 µL of diluent in wells #2 through #12.
 - a. Add 25 µL of reactive control to well #1. Mix thoroughly by filling and discharging the pipette 6 to 8 times. Transfer 25 µL to well #2. Mix as before and Transfer 25 µL to well #3. Continue this procedure through well #12. Discard 25 µL from well #12.
 - b. Add 25 µL of unsensitized particles to well #3 and 25 µL of sensitized particles to wells #4 through #12.
 - (3) A series of 4 wells are required for each patient sample and the negative control. Label wells according to specimen identifier using the letter/number cross reference system molded in the plastic.
 - (4) Place 4 drops (100 µL) of Sample Diluent in Well #1, and 1 drop (25 µL) in wells #2 through #4 using either a calibrated dropper or a micropipette.
 - (5) Using a micropipette, add 25 µL of either the positive or negative control or patient sample into the first well of the appropriately labeled set of 4 wells.
 - (6) Mix the contents of well #1 by filling and discharging the micropipette 6–8 times. Then, using the micropipette, transfer 25 µL of the diluted solution in well #1 to well #2. Mix in the same manner as before and transfer 25 µL to Well #3. Mix the contents of the well and transfer 25 µL to well #4. Mix and discard 25 µL from well #4.
 - (7) Place 1 drop (25 µL) of Unsensitized cells in well #3, and 25 µL of sensitized cells in well #4.
 - (8) Mix the contents of the well thoroughly using an automatic vibratory shaker for 30 sec.
 - (9) Cover the plate with an empty plate or microplate cover. Let stand at room temp (15 to 30°C) for 2 hours before reading. The incubation can be extended overnight without any perceptible differences in patterns.
- D. Interpretation of Results

Place the plate on a flat surface with a white background and visually observe the pattern of agglutination in each well. Observe the agglutination pattern for each patient and control well. Ensure that each of the unsensitized particle wells is nonreactive and interpret the criteria shown in Table 1.

Note: A plate viewer, with indirect lighting, may be used to aid in the visual interpretation. Carefully place the microplate on the plate viewer, being careful not to shake or bump the plate.

Table 1. Interpretation of agglutination patterns

Settling Patterns of Particles	Reading	Interpretation
Particles are concentrated in the shape of a button at the center of the well with a smooth round outer margin.	-	Nonreactive
Particles are concentrated in the shape of a compact ring with a very small "hole" in the center and a smooth round outer margin.	-	Nonreactive
Particles are concentrated in the shape of a compact ring with a "hole" in the center and a smooth round outer margin.	±	Inconclusive
Particles form a large ring with a rough multiform outer margin and peripheral agglutination, surrounded by a small red circle.	1+	Reactive
Agglutinated particles spread out uniformly covering the bottom of the well, surrounded by a red circle.	2+	
Smooth mat of particles covering less than the entire bottom of the well, and may be surrounded by a faint ring.	3+	

Smooth mat of particles covering the entire bottom of the well, edges sometimes folded.

4+

E. Recording of Data

(1) Quality Control Data

(2) Record lot number of kit, date of testing, and titer of control serum

(a) The titer of the reactive control should be $1:320 \pm 1$ doubling dilution.

The wells with unsensitized cells should be nonreactive.

(b) Analysis

A serum sample showing nonreactive with unsensitized particles, but demonstrating a reaction of $>1+$ with the sensitized cells (dilution 1:80) is interpreted as reactive.

A serum sample showing nonreactive with unsensitized cells, but demonstrating a reaction of \pm with sensitized cells is considered inconclusive or indeterminant. Retest the sample. If it is still indeterminant, request a new sample drawn at least a week after the first.

A serum sample showing a nonreactive in both unsensitized and sensitized cells is nonreactive.

Any sample showing any other combination should be repeated.

F. Replacement and Periodic Maintenance of Key Components

All pipettors should be checked, repaired, and recalibrated at least yearly.

G. Calculations

Not applicable to this procedure.

H. Special Method Notes

(1) Do not cross-contaminate reagents. Always use a new pipette tip when drawing from stock reagent bottles.

(2) Always keep the upper surface of the microplates free of excess fluid droplets to prevent possible dilution of well contents. Blot reagent overspills.

(3) Liquid reagents are clear with no precipitates.

(4) Only reagents from the same kit are used. Mixing reagents from different lot numbers of kits may give erroneous results.

9. REPORTABLE RANGE OF RESULTS

Results are reported as Reactive, Nonreactive, or Inconclusive.

10. QUALITY CONTROL (QC) PROCEDURES

A. Evaluation of TP-PA kits is the responsibility of the user.

Reagents evaluated as described here must produce results comparable to those obtained with reference reagents. All glassware used must be free of contamination, and distilled water used as diluent must be pure.

B. Evaluation Procedure

Test 10 individual serum samples of predetermined reactivity on each of 2 days. The recommended distribution is three reactive serum samples, three minimally reactive serum samples, and four nonreactive serum samples. If necessary, prepare reactive serum samples of various levels of

reactivity by diluting reactive samples with nonreactive serum samples. These pooled samples may be substituted for some of the individual serum samples.

C. Testing

The TP-PA reagents from the new and the reference lots are tested on 2 days by using reactive and nonreactive control serum samples from the new kit and the reference kit and 10 individual serum samples.

- (1) Assemble the 10 individual serum samples described above in B.
- (2) Perform the tests on reactive control, nonreactive control and individual serum specimens. Test all serum specimens in parallel, using new and reference (old) reagents.
- (3) Read and record test results.
- (4) Compare the results obtained with reference and new reagents. Determine whether new TP-PA reagents meet the criteria of acceptability.
- (5) If results between reagent lots are discordant, additional testing may be necessary.
- (6) If the new kit gives the established reactivity patterns for known controls other than the manufacturer supplied controls, further testing can continue.

D. Daily Control

- (1) Temperatures of refrigerators must be recorded daily.
- (2) At each routine test run, check expiration date on kit.
- (3) Test kit reactivity with control serum specimens of graded reactivity (high titered and low titered reactive, nonreactive controls). Use only if results fall within ± 1 doubling dilution of the titer of the reactive control.

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

If the titer of the reactive control is more than ± 1 doubling dilution pattern of agglutination for the unsensitized particles is other than, the test must be repeated.

If the controls are still out of compliance when repeated, a new kit should be used.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Serum that is excessively lipemic, hemolyzed, or contaminated may interfere with the reaction.

Serum that has been repeatedly frozen and thawed may be falsely negative in the test.

Serum or reagents that have not reached room temperature before performing the test may cause false negative reactions.

Improperly diluting the serum samples will cause erroneous results. If the sample is diluted too much, it may be falsely negative. If not diluted enough, a false-positive result may occur.

Disturbing the microplate during incubation may prevent cells from settling properly leading to erroneous results.

13. REFERENCE RANGES (NORMAL VALUES)

Not applicable to this procedure.

14. CRITICAL CALL RESULTS (PANIC VALUES)

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens must be at room temp (18–25°C) during preparation and testing. Otherwise, store the serum at <–20°C. If the sample is going to be retested within 24 hours, store at 4–8°C to avoid a freeze-thaw cycle.

16. ALTERNATE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

If a serum sample is reactive in both the unsensitized and sensitized cells, the serum should be pre absorbed with unsensitized particles.

Place 0.95 mL of unsensitized particles in a small test tube (e.g., 12 × 75 mm).

Add 50 µL of test specimen and mix thoroughly using vortex mixer and incubate at room temp for 20–30 min (mix manually 1–2 times).

Centrifuge for 5 min at 2000 rpm. Place 50 µL of supernatant (absorbed sample) to well #3.

Add 25 µL of sample diluent to well #4. Transfer 25 µL from well #3 to well #4. Mix well and discard 25µL from well #4.

Place 1 drop of unsensitized particles in well #3, and 25 µL of sensitized particles in well #4.

Incubate at room temp for 2 hours and read the agglutination pattern.

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

Not applicable to this procedure.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

We recommend that records, including QA/QC data, be retained for 2 years beyond the duration of the survey. Only numerical identifiers (e.g., NCHS ID numbers) should be used.

For the NHANES III study, residual samples are stored at <–20°C for 1 year after analysis, then returned to the NCHS serum repository at Rockville, MD.

19. Summary Statistics and QC graphs

Qualitative assays are qualitative assays with a positive, negative or borderline/indeterminate result. The absorbance or reactivity values of specimens are compared with a cutoff value that is a ratio of the negative control mean and the positive control mean. Since the controls are read as cutoff values, plots of these values are not generated for quality control purposes.

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